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(54) METHOD OF ASSAYING PROTEASES AND THIN MEMBRANE USED IN SAID METHOD

(57) A method of assaying proteases such as matrix metalloproteases comprising the steps of (1) bringing biological samples such as protease-containing tissue slices or extracts into contact with a thin membrane containing protease substrates and hard coating agents and formed on the surface of a support, and (2) detecting the traces of digestion formed on the membrane by the action of the protease with the naked eye or under a microscope.

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Description

Technical Field

5 The present invention relates to a method for measuring protease. More specifically, it relates to a method for measuring protease which enables accurate diagnosis of malignancy of cancer cells such as infiltrative and metastatic activity, degree of progress of periodontal diseases such as alveolar pyorrhea, destructive pathological conditions in rheumatoid arthritis and the like.

10 Related Art

Presence or absence of infiltration into interstitium connective tissue is one of the factors that determine difference of benignancy and malignancy of tumor. In order to clarify the pathological conditions, it is necessary to observe changes of growth dynamics of tumor cells, per se, and to find factors affecting the interaction between tumor cells and interstitium connective tissues. In particular, it has been revealed that proteases are involved in infiltration and metas-
 15 tasis of tumor cells, and accordingly, the inhibition of infiltration and metastasis of malignant tumor cells is expected by controlling proteases. Among such proteases (extracellular matrix lyases), important roles of matrix metalloproteinase (MMP), in particular, has been elucidated in the growth and infiltration of cancer cells, and arterialization (see "Molecular Mechanism of Tumor Metastasis", Ed. By T. Tsuruo, Chapter 8; K. Miyazaki, "Matrix Proteases and Infiltration/metastasis of Cancer", pp.92-107, Medical View Co., Ltd., 1993).

Periodontal diseases progress with destruction of crevicular epithelium and connective tissues which mainly consist of collagen as early nidus, and the involvement of matrix metalloproteinase has also been known in the destruction of tissues (for the involvement of proteases in the destruction of periodontal tissues and correlation of pathological conditions and proteases, see "Science of Periodontal Treatment", Ed. by M. Aono, Chapter VII, M. Shirakawa, "Pathology of Periodontal Tissues", pp.99-109, Ishiyaku Shuppan; Hasegawa et al., "Gelatinase Activity in Gingival Crevicular Fluids (GCF) of Periodontal Disease Patients", Presentation No. A-44, 37th Fall Congress of Japan Periodontal Disease Society and other).

The matrix metalloproteinases degrade extracellular substrates such as collagen, proteoglycan, laminin, fibronectin and gelatin, and the existence of eight types, e.g., MMP-1, 2, 3, 7, 9, 10, are known. Interstitial collagenase (MMP-1), a matrix metalloproteinase known for the longest, distributes over fibroblast, cartilage and other, and cleaves interstitial collagen into 1/4 and 1/3 portions. In periodontal diseases, mainly MMP-2 (gelatinase A) and MMP-9 (gelatinase B) destroy, for example, collagen type IV, laminin, fibronectin, and proteoglycan as components of periodontal tissues. The secretion of matrix metalloproteinase is strongly promoted by EGF and TGF- β as cell growth factors, and the secretion and the expression of activity are controlled by endogenous inhibitors in tissues. However, the ways of suppression
 35 of its expression have not been fully clarified when growth factors are involved.

Plasminogen activator (PA), a one of serine proteases, is another protease involved in infiltration and metastasis of tumor cells. The plasminogen activator converts plasminogen into plasmin, and plasmin formed by the action of plasminogen activator converts a prometalloproteinase into a metalloproteinase as an active form. Accordingly, it is considered that infiltration and metastasis of cancer cells are progressed or accelerated by a cascade formed between matrix metalloproteinases and plasminogen activator.
 40 metalloproteinases and plasminogen activator.

Protease may participate in destructive pathological conditions such as destruction of bone tissue and alveolar periosteum caused by alveolar pyorrhea and destruction of periost and bone tissue caused by rheumatoid arthritis, in addition to the infiltration and metastasis of cancer cells and progress of periodontal diseases (for involvement of proteases in rheumatism, see Nippon Rinsho [Japan Clinic], 50(3), pp.463-467, 1992). Therefore, the measurement of protease in cells and tissues may enable accurate diagnosis of malignancy of cancer cells on the basis of infiltrative and metastatic activity, pathological condition of periodontal diseases, and degree of progress of destructive pathological conditions such as rheumatism (for correlation between levels of infiltration of cancer cells and protease activity, see, for example, Yamagata, et al., Cancer Lett., 59, 51, 1991; Azzam, et al., J. Natl. Cancer Inst., 85, 1758, 1993; Brown, et al., Clin. Exp. Metastasis, 11, 183, 1993; Davies, et al., Br. J. Cancer, 67, 1126, 1993).

As methods for measuring protease, zymography methods where enzyme activity is determined based on the degree of substrate degradation, immunoblotting methods utilizing antibodies specific for each protease or other have been used. For example, a method is known which comprises the steps of homogenizing cancer cells or periodontosis cells, subjecting the extract to electrophoresis utilizing gelatin-containing SDS-polyacrylamide gel, staining the gel after the electrophoresis using Amido Black, followed by determining a sample as protease positive that provides a white clear band not stained. However, according to the method, the preparation of SDS-polyacrylamide gel is required for every measurement and the process takes about 30 hours before obtaining measuring results.
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Another method is available where a gel after an electrophoresis according to the SDS-PAGE technique is placed on a membrane, and after blotting procedure, enzymes are detected by using monoclonal antibodies. However, the

method also uses electrophoresis, which is the same drawback as the aforementioned method. In addition, this method has further problems that it requires skills for operation and uses expensive monoclonal antibodies. Furthermore, these methods do not achieve measurement of protease in each individual cell, but they only measure total protease in the whole tissue. Therefore, they also have a problem in failing to provide information about infiltrative and metastatic activity of individual cancer cells.

Recently, a method is proposed wherein protease activity in vascular tissues is measured based on zymography (The FASEB Journal, Vol. 9, July, pp.974-980, 1995). According to the method, casein or gelatin modified with a fluorescent compound is used as a substrate for protease, and the method comprises the steps of forming a thin membrane of agarose containing the substrate on a microscope slide, placing unfixed tissue slice (6-10 μm) on the surface of the thin membrane and incubating the slide at 37°C, and observing the digestion of the substrate by means of a fluorescence microscope (see, the protocol set out on page 975, right column, lines 6-18). Although this method has an advantage of direct measurement of protease in tissues, it requires agarose as an essential component to fix the substrate for protease on a microscope slide, which causes fluctuation in substrate digestion by protease and results in a problem of low reproducibility.

Disclosure of the Invention

An object of the present invention is to provide a convenient and accurate method for measuring protease. More specifically, the object of the present invention is to provide a method for measuring protease which can achieve prompt and accurate determination of malignancy of cancer cells such as infiltrative and metastatic activity, pathological conditions of periodontal diseases and the like, degree of progress of destructive pathological conditions such as in rheumatism, and is useful for accurate diagnosis of, for example, prognosis of cancer, degree of progress of destructive pathological conditions.

Another object of the present invention is to provide a method for measuring protease which has the characteristic features mentioned above, and is capable of accurately measuring protease derived from cancer cells or other localized in test tissues.

A still further object of the present invention is to provide a thin membrane used for the method for measuring protease mentioned above.

The inventors of the present invention conducted various studies to achieve the foregoing objects. As a result, they found that, when a slice of tissue such as cancer tissue is brought into contact with a surface of a thin membrane containing a protease substrate such as gelatin together with a hardening agent, or when exudate collected from a morbid tissue such as a tissue of a periodontal disease is dropped onto the thin membrane, proteases contained in the sample digest the thin membrane and form the traces of digestion on the surface of the thin membrane. They also found that the method had much higher reproducibility compared to the method utilizing a thin membrane containing agarose (The FASEB Journal, Vol.9, July, pp.974-980, 1995), and achieved accurate measurement of protease activity in the samples. Furthermore, it was also found that proteases expressed in individual cells can be determined by preparing histopathological preparations and the above thin membrane preparations each by using one of continuous tissue slices and comparing the resulting preparations. The present invention was achieved on the basis of these findings.

The present invention thus provides a method for measuring protease which comprises the steps of (1) bringing a sample containing protease into contact with a thin membrane which comprises a protease substrate together with a hardening agent and is formed on a surface of a support; and (2) detecting traces of digestion on the thin membrane formed by the action of the protease.

According to the second embodiment of the present invention, there is provided a method for measuring protease which comprises the step of (1) bringing one of two substantially continuous slices of a biological sample into contact with a thin membrane which comprises a protease substrate together with a hardening agent and is formed on a surface of a support; (2) detecting the trace of digestion on the thin membrane formed by the action of protease; and (3) comparing the trace of digestion with a histopathological preparation prepared from the other slice.

According to the third embodiment of the present invention, there is provided a method for measuring protease which comprises the steps of (1) bringing one of two or more substantially continuous slices of a biological sample into contact with a thin membrane which comprises a protease substrate together with a hardening agent and is formed on a surface of a support; (2) bringing the remaining slices into contact with a thin membrane which comprises a protease substrate, a hardening agent and a protease inhibitor and is formed on a surface of a support; (3) detecting the trace of digestion on each thin membrane formed by the action of protease; and (4) comparing the trace of digestion on the thin membrane used in the step (1) with the trace of digestion on the thin membrane used in the step (2). A preferred embodiment of the aforementioned method comprises, in the step (2), a step of bringing the two or more remaining slices into contact with thin membranes each containing a different protease inhibitor.

According to the fourth embodiment of the present invention, there is provided a method for measuring protease which comprises the steps of (1) bringing one of two or more substantially continuous slices of a biological sample into

contact with a thin membrane which comprises a protease substrate together with a hardening agent and is formed on a surface of a support; (2) bringing the remaining slices into contact with a thin membrane which comprises a protease substrate different from the protease substrate contained in the thin membrane used in the step (1) together with a hardening agent and is formed on a surface of a support; (3) detecting the trace of digestion on each thin membrane formed by the action of protease; and (4) comparing the trace of digestion on the thin membrane used in the step (1) with the trace of digestion on the thin membrane used in the step (2). A preferred embodiment of the aforementioned method comprises, in the step (2), a step of bringing the two or more remaining slices into contact with thin membranes each containing a different protease substrate.

According to the fifth embodiment of the present invention, there is provided a method for measuring protease which comprises the steps of (1) bringing a sample containing protease into contact with a thin membrane comprising at least the following two layers: layer (a) which contains a protease substrate, a hardening agent and a protease inhibitor and is formed on a surface of a support, and layer (b) which contains a protease substrate and a hardening agent and is laminated on layer (a); (2) detecting the trace of digestion on the thin membrane formed by the action of protease; and (3) comparing the trace of digestion on layer (a) with the trace of digestion on layer (b).

According to the sixth embodiment of the present invention, there is provided a method for measuring protease which comprises the steps of (1) bringing a sample containing protease into contact with a thin membrane comprising at least the following two layers: layer (a) which contains a protease substrate together with a hardening agent and is formed on a surface of a support, and layer (b) which contains a protease substrate different from the protease substrate contained in layer (a) together with a hardening agent and is laminated on layer (a); (2) detecting the trace of digestion on the thin membrane formed by the action of protease; and (3) comparing the trace of digestion on layer (a) with the trace of digestion on layer (b).

These methods are characterized in that a sample containing protease or a biological sample is brought into contact with a surface of a thin membrane and not with a support.

According to another aspect of the present invention, thin membranes for measuring protease defined in the aforementioned methods are provided.

According to preferred embodiments of these inventions, there are provided the aforementioned methods and thin membranes wherein the protease substrate is selected from the group consisting of collagen, gelatin, proteoglycan, fibronectin, laminin, elastin and casein; the aforementioned methods and thin membranes wherein the sample is a biological sample isolated or collected from a patient; the aforementioned methods and thin membranes wherein the biological sample is a cancer tissue slice, gingival crevicular exudate, or a destructive morbid tissue slice or an extract (for example, an extract of a rheumatoid morbid tissue or an alveolar pyorrhea tissue); the aforementioned methods and thin membranes wherein the protease is matrix metalloproteinase; the aforementioned methods and thin membranes wherein the trace of digestion is detected by a staining; the aforementioned methods wherein the detection is performed by using a thin membrane containing one or more substances selected from the group consisting of metals, metal oxides, pigments and dyes, and having a maximum transmission density of 0.01 or higher at a wavelength ranging from 400 nm to 700 nm; and the aforementioned methods and thin membranes wherein gelatin is used as the protease substrate, and the trace of digestion is stained by means of a gelatin staining using Amido Black or Coomassie Blue.

According to preferred embodiments of the thin membranes of the present invention, there are provided the aforementioned thin membranes which comprise one or more substances selected from the group consisting of metals, metal oxides, pigments and dyes, and have a maximum transmission density of 0.01 or higher at a wavelength ranging from 400 nm to 700 nm; the aforementioned thin membranes wherein the support is selected from a microscope slide and a polyethylene terephthalate film; the aforementioned thin membranes wherein an undercoat layer is provided between the support and the thin membrane; and the aforementioned thin membranes which are photographic films after light exposure, development and fixation.

According to further aspect of the present invention, there are provided methods of diagnosing a disease in which protease is involved according to the steps defined in each of the aforementioned methods. As preferred embodiments of the aforementioned methods, there are provided the methods wherein the disease is selected from the group consisting of cancer, rheumatic diseases, periodontal diseases and alveolar pyorrhea.

Best Mode for Carrying Out the Invention

The methods of measuring protease according to the aforementioned embodiments basically comprise a step of bringing a sample containing protease into contact with a thin membrane (first step), and a step of detecting the trace of digestion on the thin membrane formed by the action of the protease (second step). The thin membranes for measuring protease used in the methods are formed on a surface of a support as monolayer or multilayer, and characterized in that they contain a protease substrate and a hardening agent as essential components. Therefore, the process according to the methods of the present invention is characterized in that the sample is not come into contact with the support at the beginning of the step of contact of the sample with the thin membrane. Furthermore, since the aforemen-

tioned thin membranes contain a hardening agent and are free from a binder such as agarose, the membranes contain a high density of a protease substrate such as gelatin to be digested by protease, and the breaking of the trace of digestion is prevented by the cross-linking of the protease substrate such as gelatin. For these reasons, the methods have characteristic features of excellent sensitivity and reproducibility of protease measurement.

The term "method of measurement" herein used should be construed in its widest sense including qualitative and quantitative analysis. In the methods of the present invention, the protease substrate is digested by proteases contained in a sample, and traces of digestion are formed on the thin membrane. These traces of digestion can be detected, for example, under a microscope, optionally after a staining, to verify the presence of proteases in the sample.

Proteases as the object of the measurement according to the present invention include, for example, matrix metalloproteinase (MMP) and matrix serine protease (MSP). These enzymes are explained in detail in "Molecular Mechanism of Cancer Metastasis", Ed. By T. Tsuruo, pp.92-107, Medical View Co., Ltd., 1993. Examples of proteases particularly suitable for the methods of the present invention include, for example, matrix metalloproteinases such as interstitial collagenase (MMP-1), gelatinase A (MMP-2) and gelatinase B (MMP-9); matrix serine proteases such as plasminogen activator (PA) and the like. However, the objects of the methods of the present invention are not limited to these proteases.

The protease substrates are not particularly limited so long as they are macromolecular compounds degradable as substrates of proteases. For example, collagen, gelatin, proteoglycan, fibronectin, laminin, elastin, casein and the like may be used. Preferably, collagen, gelatin, fibronectin, elastin or casein may be used, and gelatin, fibronectin and casein are more preferably used. The aforementioned substances may be used alone as the protease substrate, or in combination of two or more of them.

By using two or more different protease substrates in combination, a type of a protease contained in a biological sample can sometimes be accurately identified. For example, a method may be employed wherein one of two or more substantially continuous slices of a biological sample are brought into contact with a thin membrane containing a protease substrate; and the remaining slice is brought into contact with a thin membrane containing a protease substrate different from the protease substrate contained in the aforementioned thin membrane; and then traces of digestion formed on each of the thin membranes are compared. According to the method, the measurement can be performed by using three or more thin membranes each containing a different protease substrate. Furthermore, for example, a method may also be employed wherein a single thin membrane is prepared which comprises a first layer containing a protease substrate and a second layer containing a protease substrate different from the protease substrate contained in the first layer; a sample containing protease is brought into contact with the thin membrane; and then traces of digestion formed on each of the layers are compared. According to the aforementioned method, it is also possible to use a thin membrane comprising laminated three or more layers each containing a different protease substrate.

By using a protease inhibitor, identification of a protease that relates to the inhibitor or determination of properties of a protease may be facilitated. Examples of the protease inhibitors include, for example, tissue inhibitor of metalloproteinase 1 (TIMP1), tissue inhibitor of metalloproteinase 2 (TIMP2), large inhibitor of metalloproteinase (LIMP), chicken inhibitor of metalloproteinase (ChIMP), opostatin, platelet factor IV (PF-4), α_2 macroglobulin, EDTA, 1,10-phenanthroline, BB94, minocycline, matristatin, SC-44463, dithiothreitol (DTT) and the like. For example, a method may be employed wherein one of two or more substantially continuous slices of a biological sample is brought into contact with a thin membrane containing a protease substrate; and the remaining slices are brought into contact with other thin membranes containing a protease substrate together with a protease inhibitor; and then traces of digestion formed on each of the thin membranes are compared. In addition, for example, a method may also be employed wherein a single thin membrane is prepared which contains a first layer containing a protease substrate and a second layer containing a protease substrate together with a protease inhibitor; a sample containing protease is brought into contact with the thin membrane; and then traces of digestion formed on each of the layers are compared. The methods utilizing protease inhibitors may further be combined with the aforementioned method utilizing two or more different protease substrates.

The hardening agent used for the manufacture of the thin membrane of the present invention has actions of promoting the curing of the thin membrane during the manufacture of the thin membrane containing the protease substrate and/or preventing the swell of the thin membrane after the formation of the thin membrane. Types of the hardening agent are not particularly limited so long as the agents have the above actions and do not substantially inhibit the reaction of protease and the protease substrate, and inorganic or organic hardening agents may be used. For example, hardening agents such as chromium salts (chromium alum, chromium acetate and other); aldehydes (formaldehyde, glyoxal, glutaraldehyde and other); N-methylol compounds (dimethylolurea, methyloldimethylhydantoin and other); dioxane derivatives (2,3-dihydroxydioxane and other); compounds exhibiting activities through the activation of carboxyl group (carbenium, 2-naphthalenesulfonate, 1,1-bispyrrolidino-1-chloro-, pyridinium, 1-morpholinocarbonyl-3-(sulfonatoaminomethyl)-and other); activated vinyl compounds (1,3-bis(vinylsulfonyl)-2-propanol, 1,2-bis(vinylsulfonyl)acetamido)-ethane, bis(vinylsulfonylmethyl) ether, vinyl polymers having vinylsulfonyl groups in side chains, 1,3,5-triacryloylhexahydro-s-triazine, bis(vinylsulfonyl)methane and other); activated halogen compounds (2,4-dichloro-6-hydroxy-s-tri-

azine, sodium salt thereof and other); mucohalogen acids (mucochloric acid, mucophenoxychloric acid and other); isoxazole compounds; dialdehyde starch; 2-chloro-6-hydroxytriazinylated gelatin may be used alone or in combination of two or more of them. Among them, vinylsulfonic acid-type hardening agents are preferred. An amount of the hardening agent is not particularly limited. For example, when gelatin is used as the protease substrate, about 0.1 to 20 mmol, more preferably about 0.3 to 10 mmol per 100 g of gelatin may be formulated from a viewpoint of detection performance.

As samples used for the method of the present invention, for example, biological samples isolated or collected from mammals including human may be used. The biological samples may be tissues or tissue exudate and the like. For example, cancer tissues from solid tumor tissues of lung cancer, stomach cancer, esophageal cancer, breast cancer, brain tumor and the like isolated or collected by surgical operation or histological examination; synovial fluid and bone tissue of rheumatoid arthritis; destructive morbid tissues such as periodontal ligament or bone tissue and exudate of alveolar pyorrhea; gingival crevicular exudate of periodontal diseases and the like may be used.

When a tissue is used as a sample, for example, a slice having a thickness of 1-10 μm , preferably 5 μm may be prepared from a sample rapidly frozen in liquid nitrogen by using an apparatus for preparing frozen sections, and then the slice may be applied to a thin membrane to bring the sample contact with the thin membrane. When synovial fluid collected from a patient of rheumatoid arthritis is used as a sample, about 5-50 μl , preferably about 20 μl of synovial fluid can be dropped onto the thin membrane. When gingival crevicular fluid of periodontal disease is used as a sample, a filter paper piece may be inserted into gingival crevice to collect about 5-10 μl of gingival crevicular fluid, and the filter paper may be applied to a thin membrane. After the collection of gingival crevicular fluid, the gingival crevicular fluid may be optionally extracted from the filter paper using distilled water or a suitable buffer (for example, 50 mM Tris-HCl, pH 7.5, 10 mM CaCl_2 , 0.2 M NaCl), and the extract may be dropped onto a thin membrane.

The thin membrane is preferably formed on a transparent or translucent support so that the presence of protease in a tissue can be observed under a microscope. Examples of such transparent or translucent support include, for example, glass and transparent or translucent plastic films made of polyethylene terephthalate, polycarbonate, polyimide, nylon, cellulose, triacetate and the like. As glass, it is preferred to use a microscope slide, and as a plastic film, it is preferred to use a polyethylene terephthalate film. However, the support is not limited to these examples, and any materials may be used so far that they can provide uniform thin membranes which are suitable for microscopic observation.

When a sample solution such as gingival crevicular fluid is dropped onto a thin membrane surface, traces of digestion may be detected along the peripheral circle of the circular applied area that is formed by the drop. In that case, detection under a microscope is not necessarily required, and accordingly, opaque supports can be used in addition to the above-explained supports. For example, paper, plastic paper, paper laminated with synthetic resin (for example, polyethylene, polypropylene, polystyrene, polyethylene naphthalate and other), metal plates (for example, plates of aluminium, aluminium alloy, zinc, iron, copper and other), paper or plastic films laminated or deposited by vaporization with the aforementioned metals and the like can be used. In the aforementioned embodiment, the support may be colored.

The thickness of the support is not particularly limited. As to glass supports, those having an usual thickness for a microscope slide (for example, approximately 2 to 3 mm) may be preferred. As to polyethylene terephthalate films, those having a thickness of about 100-250 μm , more preferably about 150-200 μm , and most preferably about 175 μm may be used. The thin membrane on the support can be formed as monolayer or multilayer, and the thin membrane should be prepared so as to have a surface as uniform as possible. For example, the thin membrane may preferably be formed so as to have a thickness of about 1-10 μm , more preferably about 4-6 μm after drying.

For the preparation of the thin membrane, for example, a protease substrate, which is dispersed in water or an organic solvent such as methylene chloride, acetone, methanol and ethanol or a mixed solvent thereof, may be splayed on a surface of a support and dried. As method for application, dip coating method, roller coating method, curtain coating method, extrusion coating method and the like can be employed. However, methods for preparing the thin membrane are not limited to these examples, and methods conventionally used for the preparation of thin membranes in the field of photographic films, for example, may be appropriately employed. When gelatin is used as the protease substrate, a type of gelatin is not particularly limited. For example, alkali extracted bovine bone gelatin, alkali extracted swine cutis gelatin, acid extracted bovine bone gelatin, phthalation-treated bovine bone gelatin, acid extracted swine cutis gelatin and the like can be used.

When a thin membrane is formed on a support, an undercoat layer may be provided between the thin membrane and the support in order to improve the adhesion of the thin membrane and the support. For example, the undercoat layer may be formed by using a polymer or a copolymer obtained by the polymerization of one or more monomers selected from vinyl chloride, vinylidene chloride, butadiene, methacrylic acid, acrylic acid, itaconic acid, maleic anhydride and the like, or a polymer such as polyethyleneimine, epoxy resin, grafted gelatin, nitrocellulose and the like. When a polyester support is used, adhesion between the support and the thin membrane may sometimes be improved by subjecting the surface of the support to corona discharge treatment, ultraviolet irradiation or glow discharge treatment instead of providing the undercoat layer.

The wording "a thin membrane formed on a surface of a support" and equivalents thereof should not be construed

to exclude those with one or more undercoat layers and/or a surface treatment of the support. Means to improve the adhesion of the thin membrane and the support are not limited to those mentioned above, and for example, those conventionally used in the field of photographic film and other can be appropriately used.

For the preparation of the thin membrane, other components such as dyes, pigments, antiseptics, stabilizers and the like may optionally be added in addition to the components mentioned above. These components may be appropriately chosen and employed so long as they do not substantially inhibit the reaction of protease and the protease substrate. For example, as the dyes, those disclosed in the Japanese Patent Unexamined Publication (KOKAI) No. (Hei)6-102624/1994 (dyes specifically represented by formula 1-1 on page 9 to formula 63 on page 47) can be used. As methods for adding dyes, those disclosed in the Japanese Patent Unexamined Publication (KOKAI) No. (Hei)5-313307/1993 (methods specifically explained from paragraph [0037] on page 11 to paragraph [0044] on page 12) can be used. The addition of a dye or a pigment may facilitate the detection of traces of digestion. For this purpose, a metal and a metal oxide may also be formulated other than the dye and the pigment. When a substance such as metals, metal oxides, dyes, and pigments is added, the thin membrane as the final product may preferably have a maximum transmission density of 0.01 or higher at a wavelength ranging from 400 nm to 700 nm. One or more of these substances may be formulated in the thin membrane, and different substances such as dyes in different colors may be added to each of the layers constituting the thin membrane. If agarose is used for the manufacture of the thin membrane, the reproducibility of protease detection may be lowered. Therefore, it is not preferred to use agarose for the manufacture of the thin membrane of the present invention.

According to the methods of the present invention, a sample containing protease is brought into contact with a thin membrane by, for example, applying a tissue slice to the thin membrane or dropping a liquid sample onto the thin membrane, and then incubation is carried out preferably in a humidified box at 37°C for a period of, for example, about 1-24 hours, preferably about 2-12 hours, more preferably about 3-6 hours for tissue slices, or about 0.5-12 hours, preferably about 1-6 hours, more preferably about 1-3 hours for liquid samples. When proteases are contained in the sample, the protease substrate in the thin membrane is degraded by the proteases, and the traces of digestion are formed on the thin membrane. The thin membrane is subjected to observation with the naked eyes or under a microscope, optionally after staining, to verify the presence of the protease. The evaluation may be performed spectrometrically by means of a spectrophotometer.

When gelatin is used as the protease substrate, it is preferred to observe traces of digestion after a gelatin staining. The gelatin staining can be performed according to a conventional method by using, for example, 1% Amido Black or Coomassie Blue. For example, a gelatin thin membrane is stained black dark-blue by the gelatin staining using Amido Black. When traces of gelatin digestion are formed on the surface of the gelatin thin membrane by protease, white spots not stained appear because gelatin is absent in the portions of the traces of digestion. A gelatin thin membrane is formed on a photographic film, and accordingly, a photographic film (for example, Neopan F, Fuji Photo Film Co., Ltd.) may be used as the thin membrane after light exposure and conventional development, fixation, rinsing, and drying treatment. In this case, traces of digestion can be observed as white portions on a black light-exposed film.

According to another embodiment of the method of the present invention, the presence of protease derived from each individual cells in a tissue can be accurately determined by preparing a continuous frozen sections from a cancer tissue and other, preparing a conventional histopathological preparation of one of two substantially continuous slices such as a hematoxylin/eosin staining slice, treating the other slice according to the method of measurement of the present invention, and comparing the results of each observation. This embodiment of method for measuring protease enables accurate determination of malignancy of individual cancer cells (infiltrative and metastatic activity) present in tissues; and definite diagnosis of the prognosis of the cancer. Furthermore, by quantitative measurement of protease in a sample of synovial fluid from rheumatoid arthritis, gingival crevicular fluid and the like, pathological conditions and the degree of progress of diseases can be accurately determined. For the quantitative measurement of protease in a sample, it is preferred to prepare a calibration curve using a standard solutions prepared beforehand.

Examples

The present invention will be explained more specifically by referring to the following examples. However, the scope of the present invention is not limited to these examples.

Example 1: Preparation of thin membrane for the measurement of protease

Alkali extracted bovine bone gelatin (10 g) was dissolved in pure water (127 g), and the solution was added with 1,2-bis(vinylsulfonylacetamido)ethane (2%, 0.8 ml) as a hardening agent. The solution was uniformly applied to a polyethylene terephthalate film provided with an undercoat layer so as to obtain a dried membrane having the thickness of about 5 μ m, and then the membrane was dried to obtain a gelatin thin membrane. The gelatin thin membrane was stored at room temperature before use.

Example 2: Preparation of thin membrane for the measurement of protease

Alkali extracted bovine bone gelatin solution was prepared in the same manner as in Example 1, and the solution was applied to a microscope slide so as to obtain a membrane having the thickness of about 6 μm after drying, and then the membrane was dried to obtain a gelatin thin membrane. Furthermore, by using each of alkali extracted swine cutis gelatin, acid extracted bovine bone gelatin, phthalation-treated bovine bone gelatin, acid extracted swine cutis gelatin (#G2625, Sigma) and acid extracted swine cutis gelatin (#G2500, Sigma) instead of the alkali extracted bovine bone gelatin, gelatin thin membranes were prepared.

10 Example 3: Measurement of proteases

As protease liquid samples, solutions each containing matrix metalloproteinase (MMP)-1, MMP-2 and MMP-9 (Yagai Co., Ltd.) at a concentration of from 2 pg/ml to 200 ng/ml were used. As biological samples, gingiva and gingival crevicular fluid (GCF) collected from periodontal disease patients and culture supernatants of pathogenic bacteria causing periodontal disease (*P. Gingivalis* #381 strain; *A. actinomycetemcomitans* Y4 strain; and *P. intermedia* ATCC 25611 strain) were used. About 10 μl of liquid samples were dropped onto each of the gelatin thin membranes obtained in Example 2, and tissue samples prepared as frozen slices of about 5 μm were applied to each of the gelatin thin membranes. The gelatin thin membranes were placed in a humidified box and incubated at 37°C for 4-16 hours, and then stained with Coomassie Blue.

As a result, portions not stained due to the gelatin digestion by protease (portions of white spots: traces of gelatin digestion) were observed in all of the gelatin thin membranes. In particular, gelatin thin membranes of the phthalation-treated bovine bone gelatin gave conspicuous gelatin digestion for liquid samples, and gelatin thin membranes of acid extracted swine cutis gelatin also gave distinct traces of gelatin digestion for liquid samples and tissue slices. As to MMP-1, MMP-2 and MMP-9, protease activities were observed at a concentration of 200 ng/ml after 4 hours, at 20 ng/ml after 8 hours, and at a concentration ranging from 20 to 200 pg/ml after 16 hours. Most of the gingival crevicular fluids required 8-16 hours until protease activity was recognized, and it was suggested that the amount of protease was in a range of from 2 pg/ml to 20 ng/ml . Protease activities in tissue slices were recognized at sites of crevicular epithelium, circumference epithelium and subepithelial connective tissue where strong inflammatory cell infiltration was observed.

30 Example 4: Measurement of protease in cancer tissues

As test tissue samples, surgical specimens of glossa epidermoid cancer, lung cancer and esophageal cancer were cut into pieces of about 0.5 cm thickness \times 2 cm width, rapidly frozen in liquid nitrogen, and then stored at -80°C. Continuous slices having a thickness of 5 μm were prepared from these samples using a frozen section preparing apparatus; and one of the slices was affixed to a microscope slide and dried. Then, the slice was fixed with 10% formalin for 5 minutes, and stained with hematoxylin/eosin in a conventional manner. The other continuous slices were affixed on the gelatin thin membranes produced in Example 2 and Example 3, and the membranes were placed in a humidified box and incubate at 37°C for 3-6 hours.

After the incubation, the gelatin thin membranes were stained with 1% Amido Black solution, dried, and microscopically examined. Portions where protease activities were expressed gave traces of gelatin digestion as white spots, whereas the other portions were in dark blue black. For all of the cancer tissues, the traces of gelatin digestion were recognized in individual cancer cells forming cancer alveolus lesions, and the cells located in peripheries of cancer alveolus lesions gave particularly distinct traces of gelatin digestion. Weak gelatin digestion was also observed in normal platycytes, and correlating to the progress of atypical epithelium growth, stronger gelatin digestion was recognized.

45 Example 5: Clinical case (buccal cavity maxillary gingiva cancer)

Cancer cells in a sample were those of low-differentiated epidermoid cancer which formed an alveolus lesion structure, and they destroyed bone tissues and showed strong infiltration. Portions of gelatin thin membrane, corresponding to the cancer cells in the cancer alveolus lesions, were digested to give traces of gelatin digestion as white spots on the gelatin thin membrane preparation, and portions of the gelatin thin membrane, corresponding to the cells located in peripheries of the cancer alveolus lesions, particularly showed distinct traces of gelatin digestion. At portions corresponding to focal inflammatory cell infiltration sites, remarkable granular traces of gelatin digestion were formed. When the cancer alveolus lesions were observed in magnification, distinct traces of gelatin digestion were recognized in the cells of growth sites located at peripheries of the cancer alveolus lesions, and granular traces of gelatin digestion were observed also at fibroblasts in interstitium adjacent to the cancer alveolus lesions.

Example 6: Clinical case (lingual cancer)

Cancer cells in a sample were those of undifferentiated epidermoid cancer, and they formed cancer alveolus lesions in various sizes. Every portions corresponding to the cells located in peripheries of the cancer alveolus lesions showed distinct traces of gelatin digestion, and the traces of gelatin digestion at sites corresponding to the cells of growth regions were particularly notable. Granular traces of gelatin digestion were observed also at portions corresponding to focal inflammatory cell infiltration sites.

Example 7: Clinical case (severe epithelium dysplasia of oral mucosa)

In a hematoxylin/eosin stained sample, severe epithelium dysplasia was recognized in epithelium, and hyperplasia of prickle cells and multiple stratification of basal cells were observed. In particular, polymorphism and atypical cell growth were observed in basal cells. On the gelatin thin membrane, distinct traces of gelatin digestion were recognized at portions corresponding to the hyperplastic prickle cell layer and the granulocyte layer, whereas only punctate traces of gelatin digestion were recognized in the basal cell layer. These results suggest that turnover of epithelial cells was very rapid, whereas basal cells infiltrated into the epithelial connective tissues.

When the hyperplasia of prickle cells and the multiple stratification of basal cells were observed in magnification, traces of gelatin digestion were recognized on portions corresponding to both of the cell layers, and in particular, gelatin was markedly digested at portions corresponding to the prickle cells and stratified basal cells. On the other hand, gelatin was less digested by basal cells in monolayer or bilayer compared to stratified basal cells. The stratified basal cells were deformed into a spindle shape, and polymorphism and heteromorphism of the cells were observed. Portions corresponding to the cells exhibiting heteromorphism and growing tendency formed distinct traces of gelatin digestion on the gelatin thin membrane.

Example 8: Measurement of protease activity in a liquid sample

About 20 μ l of synovial liquid of rheumatism patients was dropped on the gelatin thin membrane, and the membrane was incubated in a humidified box at 37°C for 1-3 hours. The gelatin thin membrane was then stained with 1% Amido Black. Distinct traces of gelatin digestion were observed at a peripheral circle of the applied circular portion on the gelatin thin membrane formed by the dropping. In particular, when a thin membrane of phthalation-treated bovine bone gelatin was used, particularly conspicuous traces of gelatin digestion were observed.

Example 9: Measurement of protease activity in periodontal diseases

Saliva and dental plaque on tooth surface were removed with pledgets as completely as possible for simple exclusion of moisture, and then a piece of Perio paper was inserted in gingival crevice and left stand for 90 seconds to allow the absorption of gingival crevicular fluid (about 5-10 μ l) in the Perio paper. The piece of Perio paper was extracted with 150 μ l of a buffer (50 mM Tris-HCl, pH 7.5, 10 mM CaCl_2 , 0.2 M NaCl), and protease was measured according to the same method as Example 3 using the extract as a sample solution. As a result, distinct traces of gelatin digestion were observed along the peripheral circle of the applied circular portion on the gelatin thin membrane formed by the dropping of the gingival crevicular fluid.

Example 10: Preparation of the thin membrane for the measurement of protease

Alkali extracted bovine bone gelatin (15 g) was dissolved in pure water (123 g), and the solution was added with 1,2-bis(vinylsulfonylacetyl)ethane (4% aqueous solution, 0.6 ml) as a hardening agent. The solution was uniformly applied to an object glass by a wire bar coater so as to obtain a dried membrane having the thickness of about 7 μ m, and then the membrane was dried to obtain a gelatin thin membrane (single layer thin membrane: Sample 101). The thin membrane was stored at room temperature before use. By using the protease substrates shown in Table 1 instead of the alkali extracted bovine bone gelatin, and changing or supplementing the hardening agent, the additive, and the support as shown in Table 1, Samples 102-130 were produced in the same manner as Sample 101. Upon application, an application aid was used as required. In the table, Dye 1 represents Amido Black, and Pigment 1 represents copper phthalocyanine.

Table I

Sample No.	Protease substrate		Hard coating agent		Additive		Support Content
	Content	Membrane thickness	Content	Applied amount	Content	Applied amount	
101	Alkali extracted bovine bone gelatin	7.0 μ m	1,2-Bis(vinylsulfonyl)-	0.60	-		Microscope slide
102	Acid extracted bovine bone gelatin	7.0 μ m	acetamido)ethane	g/m ²	-		Microscope slide
103	Phthalation-treated bovine bone gelatin	7.0 μ m		g/m ²	-		Microscope slide
104	Alkali extracted bovine bone gelatin, Bloom value: 300	7.0 μ m		g/m ²	-		Microscope slide
105	Alkali extracted bovine bone gelatin, Bloom value: 150	7.0 μ m		g/m ²	-		Microscope slide
106	Alkali extracted swine cutis gelatin	7.0 μ m		g/m ²	-		Microscope slide
107	Acid extracted swine cutis gelatin G2625	7.0 μ m		g/m ²	-		Microscope slide
108	Acid extracted swine cutis gelatin G2500	7.0 μ m		g/m ²	-		Microscope slide
109	Acid extracted swine cutis gelatin, Bloom value: 300	7.0 μ m		g/m ²	-		Microscope slide
110	Acid extracted swine cutis gelatin, Bloom value: 150	7.0 μ m		g/m ²	-		Microscope slide
111	Alkali deliming-treated bovine bone gelatin	7.0 μ m		g/m ²	-		Microscope slide
112	Acid extracted swine cutis gelatin	7.0 μ m		g/m ²	-		Microscope slide
113	Alkali extracted bovine bone gelatin, α -chain: 50%	7.0 μ m		g/m ²	-		Microscope slide
114	Acid extracted swine cutis gelatin, α -chain: 50%	7.0 μ m		g/m ²	-		Microscope slide
115	Casein	7.0 μ m		g/m ²	-		Microscope slide
116	Acid extracted swine cutis gelatin G2625	7.0 μ m		g/m ²	-		Microscope slide
117	Acid extracted swine cutis gelatin G2625	7.0 μ m		g/m ²	-		Microscope slide
118	Acid extracted swine cutis gelatin G2625	7.0 μ m		g/m ²	-		PET

Table 1 (continued)

119	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Carbon black	1.50 g/m ²	PET
120	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Dye 1	1.50 g/m ²	PET
121	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Pigment 1	1.50 g/m ²	PET
122	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Buffer (Tris-HCl)	0.15 g/m ²	PET
123	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Inhibitor 1 (TIMP1)	0.15 g/m ²	PET
124	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Inhibitor 2 (TIMP2)	0.15 g/m ²	PET
125	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Inhibitor 3 (EDTA)	0.15 g/m ²	PET
127	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	-		PET
128	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	-		PET
129	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	-		CTA
130	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	-		PE laminated paper

Example 11: Preparation of thin membrane for protease assay

A collagen I solution (Wako Pure Chemical Industries, 3 mg/ml) was uniformly applied to a microscope slide by a wire bar coater so as to obtain a dried membrane having the thickness of about 1 μm , and then the membrane was dried to obtain a gelatin thin membrane (single layer thin membrane: Sample 131). The thin membrane was stored at room temperature before use. By using the protease substrates shown in Table 2 instead of collagen I, and changing or supplementing the hardening agent, the additive, and the support as shown in Table 2, Samples 132-160 were produced in the same manner as Sample 131. Upon application, an application aid was used as required.

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Table 2

Sample No.	Protease substrate		Hard coating agent		Additive		Support Content
	Content	Membrane thickness	Content	Applied amount	Content	Applied amount	
131	Collagen I	7.0 μm	1,2-Bis(vinylsulfonyl)-acetamidoethane	0.60 g/m^2	-		Microscope slide
132	Collagen II	7.0 μm		0.60 g/m^2	-		Microscope slide
133	Collagen III	7.0 μm		0.60 g/m^2	-		Microscope slide
134	Collagen IV	7.0 μm		0.60 g/m^2	-		Microscope slide
135	Collagen V	7.0 μm		0.60 g/m^2	-		Microscope slide
136	Collagen VI	7.0 μm		0.60 g/m^2	-		Microscope slide
137	Collagen VII	7.0 μm		0.60 g/m^2	-		Microscope slide
138	Collagen VIII	7.0 μm		0.60 g/m^2	-		Microscope slide
139	Collagen IX	7.0 μm		0.60 g/m^2	-		Microscope slide
140	Collagen X	7.0 μm		0.60 g/m^2	-		Microscope slide
141	Alkali extracted bovine bone gelatin	7.0 μm		0.60 g/m^2	-		Microscope slide
142	Proteoglycan	7.0 μm		0.60 g/m^2	-		Microscope slide
143	Fibronectin	7.0 μm		0.60 g/m^2	-		Microscope slide
144	Laminin	7.0 μm		0.60 g/m^2	-		Microscope slide
145	Casein	7.0 μm		0.60 g/m^2	-		Microscope slide
146	Collagen IV	7.0 μm		0.10 g/m^2	-		Microscope slide
147	Collagen IV	7.0 μm		0.30 g/m^2	-		PET
148	Collagen IV	7.0 μm		1.20 g/m^2	-		PET

Table 2 (continued)

149	Collagen IV	7.0 μ m	0.60 g/m ²	Carbon black	1.50 g/m ²	PET
150	Collagen IV	7.0 μ m	0.60 g/m ²	Dye 1	1.50 g/m ²	PET
151	Collagen IV	7.0 μ m	0.60 g/m ²	Pigment 1	1.50 g/m ²	PET
152	Collagen IV	7.0 μ m	0.60 g/m ²	Buffer (Tris-HCl)	0.15 g/m ²	PET
153	Collagen IV	7.0 μ m	0.60 g/m ²	Inhibitor 1 (TIMP1)	0.15 g/m ²	PET
154	Collagen IV	7.0 μ m	0.60 g/m ²	Inhibitor 2 (TIMP2)	0.15 g/m ²	PET
155	Collagen IV	7.0 μ m	0.60 g/m ²	Inhibitor 3 (EDTA)	0.15 g/m ²	PET
157	Collagen IV	7.0 μ m	0.60 g/m ²			PET
158	Collagen IV	7.0 μ m	0.60 g/m ²			PET
159	Collagen IV	7.0 μ m	0.60 g/m ²			CTA
160	Collagen IV	7.0 μ m	0.60 g/m ²			PE laminated paper

Samples 161-170 were prepared in the same manner as Samples 121-130, except that a slide coater was used instead of the wire bar coater for application. As drying conditions, a process is applied wherein the samples was optionally once cooled to 10°C as required, and dried at ordinary temperature and humidity.

Example 12: Protease activity assay

About 10 μ l of the MMP-2 described in Example 3 was used as a protease liquid sample, and the solution was dropped onto each of the thin membranes obtained in Example 11. The thin membranes were placed in a humidified box, and incubated at 37°C for 4-16 hours. Then, transparent samples were stained with an Amido Black solution. As the evaluation of the activity, (1) observation with the naked eyes, (2) the density measurement of microportions by microdensitometry, and (3) the measurement of a membrane thickness using a contact membrane thickness measuring apparatus were performed for each of the samples. The results are shown in Table 3.

Table 3

Sample No.	Traces of digestion	Optical density	Membrane thickness (μ m)
101	Present	0.08	0.4
102	Present	0.08	0.4
103	Present	0.04	0.2
104	Present	0.08	0.4
105	Present	0.02	0.0
106	Present	0.04	0.4
107	Present	0.02	0.0
108	Present	0.02	0.0
109	Present	0.04	0.4
110	Present	0.02	0.0
111	Present	0.04	0.4
112	Present	0.04	0.4
113	Present	0.02	0.2
114	Present	0.02	0.2
115	Present	0.08	0.4
116	Present	0.02	0.0
117	Present	0.02	0.0
118	Present	0.10	0.5
119	Present	0.02	0.0
120	Present	0.02	0.0
121	Present	0.02	0.0
122	Present	0.02	0.0
123	Absent	4.50	7.0

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	124	Absent	4.50	7.0
	125	Absent	4.50	7.0
5	127	Present	0.02	0.0
	128	Present	0.02	0.0
	129	Present	0.02	0.0
	130	Present	0.02	0.0
	131	Present	0.08	0.4
10	132	Present	0.08	0.4
	133	Present	0.04	0.2
	134	Present	0.08	0.4
	135	Present	0.02	0.0
15	136	Present	0.04	0.4
	137	Present	0.02	0.0
	138	Present	0.02	0.0
	139	Present	0.04	0.4
	140	Present	0.02	0.0
20	141	Present	0.04	0.4
	142	Present	0.04	0.4
	143	Present	0.02	0.2
	144	Present	0.02	0.2
25	145	Present	0.08	0.4
	146	Present	0.02	0.0
	147	Present	0.02	0.0
	148	Present	0.10	0.5
	149	Present	0.02	0.0
30	150	Present	0.02	0.0
	151	Present	0.02	0.0
	152	Present	0.02	0.0
	153	Absent	4.50	7.0
	154	Absent	4.50	7.0
35	155	Absent	4.50	7.0
	157	Present	0.02	0.0
	158	Present	0.02	0.0
	159	Present	0.02	0.0
	160	Present	0.02	0.0
40	Control	Absent	4.50	7.0
	(untreated)			

45 In all of the thin membranes free from the protease inhibitors, white spots where the protease substrate was digested by the protease (traces of digestion) were observed, and optical density and membrane thickness of these portions were reduced compared to the surrounding portions. On the other hand, in the samples added with the protease inhibitors, traces of digestion were not observed because the protease activity was inhibited. Samples 160-170 prepared by using a slide coater gave the same results as the samples prepared by a bar coater.

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Example 13: Measurement of protease activity.

Sample 101 obtained in Example 11 was subjected to the measurement to obtain a calibration curve according to the same method as Example 12 by using solutions containing matrix metalloproteinase (MMP)-1 at a concentration of 55 from 2 pg/ml to 200 ng/ml as protease liquid samples. The results are shown in Table 4.

Table 4

Amount of MMP added (g/ml)	Traces of digestion	Optical density	Membrane thickness (μm)
10^{-7}	Present	0.2	0.1
10^{-8}	Present	0.2	0.1
10^{-9}	Present	0.2	0.1
10^{-10}	Present	1.0	0.5
10^{-11}	Slightly present	4.0	6.0
10^{-12}	Absent	4.5	7.0
No addition	Absent	4.5	7.0

The results of Table 4 were plotted in a graph to obtain the concentration of metalloproteinase (MMP)-1 that gave the optical density of the traces of digestion (2.6) when biological samples were used. As a result, it was found that the concentration of the protease in the biological samples was about 40 pg/ml.

Example 14: Protease activity assay

Biological samples of gingiva, gingival crevicular fluid, and periodontal tissue samples collected from periodontal disease patients were affixed onto Samples 101-160 of the thin membranes as about 5 μm frozen slices. The thin membranes were placed in a humidified box, and incubated at 37°C for 4-16 hours. Then, transparent samples were stained with an Amido Black solution. For the portions of the samples where the activity was clearly observed, evaluation of the activity was performed by (1) observation with the naked eyes, (2) the density measurement of microportions by microdensitometry, and (3) the measurement of a membrane thickness using a contact membrane thickness measuring apparatus. The results are shown in Table 5.

Table 5

	Sample No.	Traces of digestion	Optical density	Membrane thickness (μm)
5	101	Present	0.10	0.6
	102	Present	0.10	0.6
	103	Present	0.06	0.4
	104	Present	0.10	0.6
	105	Present	0.02	0.0
10	106	Present	0.04	0.4
	107	Present	0.02	0.0
	108	Present	0.02	0.0
	109	Present	0.04	0.4
	110	Present	0.02	0.0
15	111	Present	0.04	0.4
	112	Present	0.04	0.4
	113	Present	0.02	0.2
	114	Present	0.02	0.2
	115	Present	0.08	0.4
20	116	Present	0.02	0.0
	117	Present	0.02	0.0
	118	Present	0.16	0.8
	119	Present	0.02	0.0
	120	Present	0.02	0.0
25	121	Present	0.02	0.0
	122	Present	0.02	0.0
	123	Absent	4.50	7.0
	124	Absent	4.50	7.0
	125	Absent	4.50	7.0
	127	Present	0.02	0.0
30	128	Present	0.02	0.0
	129	Present	0.02	0.0
	130	Present	0.02	0.0
	131	Present	0.12	0.6
	132	Present	0.08	0.5
35	133	Present	0.06	0.4
	134	Present	0.12	0.6
	135	Present	0.02	0.0
	136	Present	0.04	0.4
	137	Present	0.02	0.0
40	138	Present	0.02	0.0
	139	Present	0.04	0.4
	140	Present	0.02	0.0
	141	Present	0.04	0.4
	142	Present	0.04	0.4
45	143	Present	0.02	0.2
	144	Present	0.02	0.2
	145	Present	0.10	0.5
	146	Present	0.02	0.0
	147	Present	0.02	0.0
	148	Present	0.16	0.8
50	149	Present	0.02	0.0
	150	Present	0.02	0.0
	151	Present	0.02	0.0

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	152	Present	0.02	0.0
	153	Absent	4.50	7.0
5	154	Absent	4.50	7.0
	155	Absent	4.50	7.0
	157	Present	0.02	0.0
	158	Present	0.02	0.0
	159	Present	0.02	0.0
10	160	Present	0.02	0.0
	Control	Absent	4.50	7.0
	(untreated)			

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In the method wherein frozen slices of biological samples are directly placed, traces of digestion of the protease substrate by the protease (portions of white spaces) were also formed on each thin membrane, and optical density and membrane thickness of these portions were reduced compared to surrounding portions.

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Example 15: Preparation of thin membrane for protease assay

Alkali extracted bovine bone gelatin (15 g) was dissolved in pure water (123 g), and the solution was added with 1,2-bis(vinylsulfonylacetamido)ethane (4% aqueous solution, 0.6 ml) as a hardening agent. The solution was uniformly applied to a microscope slide by a wire bar coater so as to obtain a dried membrane having the thickness of about 7 μm , and then the membrane was dried to obtain a thin membrane. Alkali extracted bovine bone gelatin (15 g) was dissolved in pure water (123 g), and the solution was added with 1,2-bis(vinylsulfonylacetamido)ethane (4% aqueous solution, 0.6 ml) as a hardening agent and polymethyl methacrylate particles (average diameter; 2 μm). This solution was uniformly applied to the surface of the already prepared dried thin membrane by a wire bar coater so as to obtain a dried membrane having the thickness of about 7 μm , and then the membrane was dried to obtain a thin membrane (multiple-layer thin membrane: Sample 301). The thin membranes were stored at room temperature before use. Those thin membranes shown in Table 6 were prepared by changing or supplementing the protease substrate and the hardening agent. For the application, application aids and viscosity modifiers were used as required. As Blue dye 1, Green dye 1 and Red dye 1 specified in the table, the following compounds were used, respectively.

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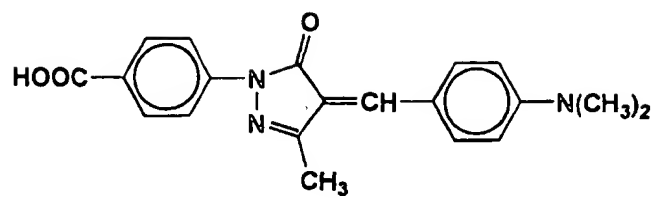
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(Blue dye 1)

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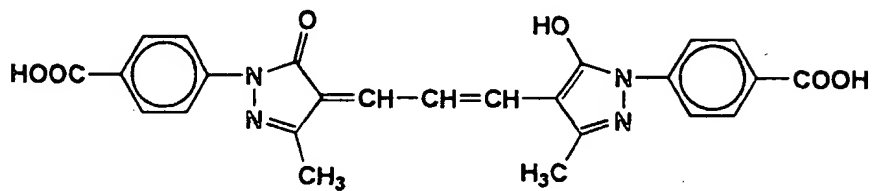
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(Green dye 1)

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(Red dye1)

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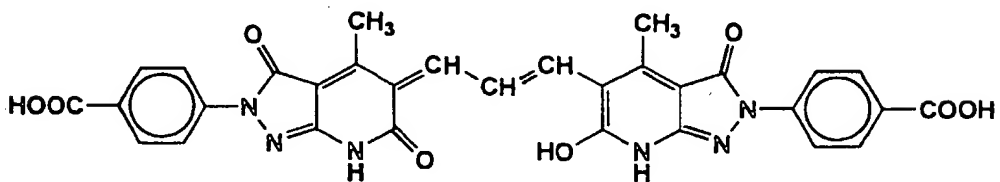


Table 6

Sample No.	First layer		Hard coating agent		Additive	
	Content	Membrane thickness	Content	Applied amount	Content	Applied amount
301	Collagen I	7.0 μ m	1,2-Bis(vinylsulfonyl)- acetamidobethane →	0.60 g/m ²	Red dye 1	1.50 g/m ²
302	Collagen I	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
303	Collagen II	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
304	Collagen III	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
305	Collagen IV	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
306	Collagen V	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
307	Collagen VI	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
308	Collagen VII	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
309	Collagen VIII	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
310	Collagen IX	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
311	Collagen X	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
312	Proteoglycan	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
313	Fibronectin	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
314	Laminin	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
315	Casein	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
316	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Inhibitor 1 (TIMP1), Red dye 1	0.15 g/m ² , 1.50 g/m ²
317	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Inhibitor 2 (TIMP2), Red dye 1	0.15 g/m ² , 1.50 g/m ²
318	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Inhibitor 3 (EDTA), Red dye 1	0.15 g/m ² , 1.50 g/m ²
322	Collagen III	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
323	Collagen IV	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
324	Collagen V	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
325	Collagen VII	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
326	Collagen IV	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
327	Proteoglycan	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
328	Fibronectin	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
329	Laminin	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²
330	Casein	7.0 μ m		0.60 g/m ²	Red dye 1	1.50 g/m ²

Table 6 (Cont.)

Sample No.	Second layer		Membrane thickness	Hard coating agent		Additive	
	Content			Content	Applied amount	Content	Applied amount
301	Alkali extracted bovine bone gelatin		7.0 μ m	1,2-Bis(vinylsulfonyl-acetamido)ethane →	0.60 g/m ²	Polymethyl methacrylate particles	0.01 g/m ²
302	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
303	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
304	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
305	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
306	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
307	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
308	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
309	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
310	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
311	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
312	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
313	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
314	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
315	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
316	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
317	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	0.15 g/m ² , 1.50 g/m ²
318	Acid extracted swine cutis gelatin G2625		7.0 μ m		0.60 g/m ²	Green dye 1	0.15 g/m ² , 1.50 g/m ²
322	Fibronectin		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
323	Laminin		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
324	Casein		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
325	Collagen II		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
326	Collagen III		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
327	Collagen IV		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
328	Collagen V		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
329	Collagen VII		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²
330	Collagen IV		7.0 μ m		0.60 g/m ²	Green dye 1	1.50 g/m ²

Table 6 (Cont.)

Sample No.	Third layer		Hard coating agent		Additive	
	Content	Membrane thickness	Content	Applied amount	Content	Applied amount
322	Acid extracted swine cutis gelatin G2625	7.0 μ m	1,2-Bis(vinylsulfonyl-acetamido)ethane →	0.60 g/m ²	Blue dye 1	1.50 g/m ²
323	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Blue dye 1	1.50 g/m ²
324	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Blue dye 1	1.50 g/m ²
325	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Blue dye 1	1.50 g/m ²
326	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Blue dye 1	1.50 g/m ²
327	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Blue dye 1	1.50 g/m ²
328	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Blue dye 1	1.50 g/m ²
329	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Blue dye 1	1.50 g/m ²
330	Acid extracted swine cutis gelatin G2625	7.0 μ m		0.60 g/m ²	Blue dye 1	1.50 g/m ²

Samples 331-360 were prepared in the same manner as the preparation of Samples 301-330, except that a slide coater was used instead of the wire bar coater for application. As drying conditions, a process is applied wherein the samples was optionally once cooled to 10°C as required, and dried at ordinary temperature and humidity.

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Example 16: Protease activity assay

Protease activity assay was performed in the same manner as Example 12. The results are shown in Table 7. From these results, it was found that the protease activity was detectable with extreme distinctness by means of the multiple-layer thin membranes. Samples 331-360 prepared by using a slide coater gave the same results as the samples prepared by a bar coater.

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Table 7

Sample No.	Traces of digestion			Optical density			Membrane thickness		
	First layer	Second layer	Third layer	First layer	Second layer	Third layer	First layer	Second layer	Third layer
301	Present	Present		0.12	0.02		0.6 μ m	0.6 μ m	
302	Present	Present		0.08	0.04		2.0 μ m	0.5 μ m	
303	Present	Present		0.06	0.02		1.0 μ m	0.4 μ m	
304	Present	Present		0.12	0.02		3.0 μ m	0.6 μ m	
305	Present	Present		0.02	0.04		4.0 μ m	0.0 μ m	
306	Present	Present		2.02	0.02		2.0 μ m	0.4 μ m	
307	Present	Present		3.50	0.04		2.0 μ m	0.0 μ m	
308	Present	Present		1.50	0.04		1.0 μ m	0.0 μ m	
309	Present	Present		3.50	0.02		3.0 μ m	0.4 μ m	
310	Present	Present		2.02	0.02		2.0 μ m	0.0 μ m	
311	Present	Present		3.50	0.04		1.0 μ m	0.4 μ m	
312	Present	Present		1.50	0.02		3.0 μ m	0.4 μ m	
313	Present	Present		3.50	0.02		4.0 μ m	0.2 μ m	
314	Present	Present		2.50	0.04		2.0 μ m	0.2 μ m	
315	Present	Present		0.10	0.02		0.5 μ m	0.5 μ m	
316	Absent	Present		4.50	0.04		7.0 μ m	0.0 μ m	
317	Absent	Present		4.50	0.04		7.0 μ m	0.0 μ m	
318	Absent	Present		4.50	0.02		7.0 μ m	0.0 μ m	
322	Present	Present	Present	2.02	0.02	0.04	3.0 μ m	0.0 μ m	0.0 μ m
323	Present	Present	Present	3.50	2.02	0.02	3.0 μ m	2.0 μ m	0.4 μ m
324	Present	Present	Present	1.50	3.50	0.04	2.0 μ m	1.0 μ m	0.4 μ m
325	Present	Present	Present	3.50	1.50	0.02	1.0 μ m	3.0 μ m	2.0 μ m
326	Present	Present	Present	2.50	3.50	0.02	3.0 μ m	2.0 μ m	0.2 μ m
327	Present	Present	Present	2.04	2.50	0.04	4.0 μ m	1.0 μ m	0.5 μ m
328	Present	Present	Present	1.23	0.02	0.02	2.0 μ m	3.0 μ m	0.0 μ m
329	Present	Present	Present	3.12	0.02	0.04	4.0 μ m	4.0 μ m	0.0 μ m
330	Present	Present	Present	2.02	0.02	0.04	3.0 μ m	2.0 μ m	0.8 μ m
Untreated, Staining alone	Absent	Absent	Absent	4.50	4.50	4.50	7.0 μ m	7.0 μ m	7.0 μ m

Example 17: Preparation of thin membrane for measuring protease and the measurement of protease activity

Alkali extracted bovine bone gelatin (15 g) was dissolved in pure water (123 g), and the solution was added with 1,2-bis(vinylsulfonylacetamido)ethane (4%, 0.6 ml) as a hardening agent. Samples 501-530 were prepared in the same manner as in the preparation of Samples 331-360, except that the above solution was applied as a back layer by using a slide coater, and dried to obtain thin membranes. Samples 531-560 were prepared by applying a polymer latex layer on the back layer of Sample 501-530, and drying the layer, the measurements of protease activity using these samples in the same manner as the aforementioned measurements using Samples 331-360 gave similar results. No curling was occurred in these samples, and the membranes were excellent in handling property.

Example 18: Measurement of protease using a photographic film

A Neopan F (monochrome film for photography, Fuji Photo Film Co., Ltd.) was completely exposed to light, and then the film was developed, fixed, rinsed and dried in a conventional manner. The photographic film was used as a gelatin thin membrane for protease measurement, and the measurement of protease activity was performed in the same manner as Example 4 by affixing slices of the cancer tissues prepared in Example 4 on this film. As a result, portions where protease was absent were remained black, whereas traces of digestion similar to those observed on the stained thin membranes prepared in Example 4 were observed as white spots. However, detection sensitivity of the film was lower than the thin membranes produced in Examples 1, 2, 10, 11, 15 and 17.

Example 19: Measurement of protease utilizing an emulsion for radiography and results of the measurement (Comparative example)

Biological samples of gingiva, gingival crevicular fluid, and periodontal tissue samples collected from periodontal disease patients were affixed onto a microscope slide as about 5 μ m frozen slices. Emulsion for radiography NRM2 or NRH2 (KONICA CORPORATION) diluted with water was applied on the slices, and dried to form thin membranes. The thin membranes were placed in a humidified box, and incubated at 37°C for 4-16 hours. Then, the membranes were stained with Amido Black or subjected to a monochromic development. As a result, almost no protease activity was detected. Trace of activity was observed in samples incubated for 14 hours and subjected to monochromic development, however, indication of the activity area was very ambiguous, and their protease detection performance was far inferior to that of the thin membranes of the present invention.

Industrial Applicability

The methods of the present invention are characterized in that they enable accurate and convenient measurement of protease derived from specific lesions localized in tissues or individual cells in tissues, and in addition, determination can be completed in a short period of time. Therefore, the methods of the present invention are useful for accurate diagnosis of malignancy of cancer cells such as infiltration and metastasis activities, degree of progress of periodontal disease such as periodontitis, destructive pathological conditions of rheumatism, alveolar pyorrhea and the like. According to the methods of the present invention, protease activity can be measured from extremely small amount of samples, and thin membranes after the measurement may be stored permanently as fixed preparations.

Claims

1. A method for measuring protease which comprises the steps of:

- (1) bringing a sample containing protease into contact with a thin membrane which comprises a protease substrate together with a hardening agent and is formed on a surface of a support; and
- (2) detecting the trace of digestion formed on the thin membrane by the action of protease.

2. A method for measuring protease which comprises the steps of:

- (1) bringing one of two substantially continuous slices of a biological sample into contact with a thin membrane which comprises a protease substrate together with a hardening agent and is formed on a surface of a support;
- (2) detecting the trace of digestion formed on the thin membrane by the action of protease; and
- (3) comparing the trace of digestion with a histopathological preparation prepared from the other slice.

3. A method for measuring protease which comprises the steps of:

- (1) bringing one of two or more substantially continuous slices of a biological sample into contact with a thin membrane which comprises a protease substrate together with a hardening agent and is formed on a surface of a support;
 - (2) bringing the remaining slices into contact with a thin membrane which comprises a protease substrate, a hardening agent, and a protease inhibitor and is formed on a surface of a support;
 - (3) detecting traces of digestion formed on the thin membranes by the action of protease; and
 - (4) comparing the trace of digestion on the thin membrane used in the step (1) with the trace of digestion on the thin membrane used in the step (2).
4. A method for measuring protease which comprises the steps of:
- (1) bringing one of two or more substantially continuous slices of a biological sample into contact with a thin membrane which comprises a protease substrate together with a hardening agent and is formed on a surface of a support;
 - (2) bringing the remaining slices into contact with a thin membrane which comprises a protease substrate different from the protease substrate contained in the thin membrane used in the step (1) together with a hardening agent and is formed on a surface of a support;
 - (3) detecting traces of digestion formed on the thin membranes by the action of protease; and
 - (4) comparing the trace of digestion on the thin membrane used in the step (1) with the trace of digestion on the thin membrane used in the step (2).
5. A method for measuring protease which comprises the steps of:
- (1) bringing a sample containing protease into contact with a thin membrane which comprises at least the following two layers: layer (a) which contains a protease substrate, a hardening agent, and a protease inhibitor and is formed on a surface of a support, and layer (b) which contains a protease substrate and a hardening agent and is laminated on the layer (a);
 - (2) detecting traces of digestion formed on the thin membrane by the action of protease; and
 - (3) comparing the trace of digestion on the layer (a) with the trace of digestion on the layer (b).
6. A method for measuring protease which comprises the steps of:
- (1) bringing a sample containing protease into contact with a thin membrane which comprises at least the following two layers: layer (a) which contains a protease substrate together with a hardening agent and is formed on a surface of a support, and layer (b) which contains a protease substrate different from the protease substrate contained in the layer (a) together with a hardening agent and is laminated on the layer (a);
 - (2) detecting traces of digestion formed on the thin membrane by the action of protease; and
 - (3) comparing the trace of digestion on the layer (a) with the trace of digestion on the layer (b).
7. The method of any one of claims 1 to 6 wherein the protease substrate is selected from the group consisting of collagen, gelatin, proteoglycan, fibronectin, laminin, elastin, and casein.
8. The method of any one of claims 1 to 7 wherein the sample is a biological sample isolated or collected from a patient.
9. The method of any one of claims 1 to 8 wherein the detection is performed by using a thin membrane containing one or more substances selected from the group consisting of metals, metal oxides, pigments and dyes and having a maximum transmission density of 0.01 or higher at a wavelength ranging from 400 nm to 700 nm.
10. The method of any one of claims 1 to 9 wherein the protease is matrix metalloproteinase.
11. A thin membrane for measuring protease which contains a protease substrate together with a hardening agent and is formed on a surface of a support.
12. The thin membrane of claim 11 which comprises at least the following two layers: layer (a) which comprises a protease substrate, a hardening agent and a protease inhibitor and is formed on a surface of a support, and layer (b) which contains a protease substrate together with a hardening agent and is laminated on the layer (a).

13. The thin membrane of claim 11 which comprises at least the following two layers: layer (a) which comprises a protease substrate together with a hardening agent and is formed on a surface of a support, and layer (b) which comprises a protease substrate different from the protease substrate contained in the layer (a) together with a hardening agent and is laminated on the layer (a).

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14. The thin membrane of any one of claims 11 to 13 which comprise one or more substances selected from the group consisting of metals, metal oxides, pigments and dyes and have a maximum transmission density of 0.01 or higher at a wavelength ranging from 400 nm to 700 nm;

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15. The thin membrane of any one of claims 11 to 14 wherein the support is selected from a microscope slide and polyethylene terephthalate film.

16. The thin membrane of any one of claims 11 to 15 wherein an undercoat layer is provided between the support and the thin membrane.

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17. A method of diagnosing a disease involving protease which comprises steps of:

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- (1) bringing a biological sample isolated or collected from a patient into contact with a thin membrane which comprises a protease substrate together with a hardening agent and is formed on a surface of a support; and
- (2) detecting the trace of digestion formed on the thin membrane by the action of protease.

18. The method of 17 wherein the disease is selected from the group consisting of cancer, rheumatic diseases, periodontal diseases and alveolar pyorrhea.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP97/00588

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl ⁶ C12Q1/37		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) Int. Cl ⁶ C12Q1/37		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) BIOSIS PREVIEWS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SU, 1178761, A (All-union order-of the-Red-Banner Scientific Research Institute for Plant Protection), September 15, 1985 (15. 09. 85) (Family: none)	1, 7, 9, 11, 14-16 8, 10, 17, 18
Y	JP, 2-2398, A (Block Drag Corp.), January 8, 1990 (08. 01. 90) & EP, 319334, A & AU, 8825954, A & US, 4931386, A	8, 10, 17, 18
Y	ANALYTICAL BIOCHEMISTRY, Vol. 104, No. 1, (1980), B. Jonson-Wint "A Quantitative Collagen Film Collagenase Assay for Large Numbers of Samples" p. 175-181	8, 10, 17, 18
Y	ANALYTICAL BIOCHEMISTRY, Vol. 76, No. 2, (1976), R. Levenson "A Collagen Film Microassay for Tissue Collagenase" p. 579-588	8, 10, 17, 18
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search May 20, 1997 (20. 05. 97)		Date of mailing of the international search report May 27, 1997 (27. 05. 97)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
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